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PROTOCOL TO DETERMINE THE ACUTE LETHALITY OF LIQUID EFFLUENTS TO FISH

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by

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INTRODUCTION

The following fish toxicity test protocol has been developed to support the Ministry's overall goal to ensure that surface waters in Ontario are of a quality which is satisfactory for aquatic life. The test is designed to identify industrial discharges acutely lethal to aquatic organisms as referred to in Policy 3 of the Ministry's publication "Water Management Goals, Policies, Objectives and Implementation Procedures". This toxicity test may also be used to assess whether materials or contaminants discharged into receiving waters impair the quality of that water or cause injury to animal life in assessing possible violations of the Ontario Water Resources Act, Section 16(1) and the Environmental Protection Act, Section 14(1a, b).

Biological tests are essential to determine whether the quality of water is satisfactory for aquatic life. The following biological test is capable of assessing the combined effects of toxicants in an effluent. This identifies whether an acutely lethal condition exists even when toxic levels of individual components have not been exceeded.

The following test is based on many years of toxicity test assessment and practice within the Ministry. The rainbow trout (_Salmo gairdneri_) has been selected as the test species in this protocol for many practical and scientific reasons. This species has been extensively studied in many laboratories. Its culture, physiology, pathology, disease association and sensitivity to industrial pollutants is well documented.

Any compound, mixture of compounds or liquid waste sample tested according to the following protocol that produces a 96-hour LC50* of less than 100% concentration shall be considered acutely lethal to fish.

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*96-hour LC50 (lethal concentration to 50%) concentration of effluent sample that is lethal to 50% of the fish exposed to that concentration over a 96-hour period.

PROCEDURE

a) Effluent Sampling and Handling Requirements

The sample must be considered as representative of the effluent by the Ontario Ministry of the Environment. The effluent sample must be collected, transported and stored in sealed clean non-toxic polyethylene containers from which air has been excluded by complete filling. The sample should not be aerated during storage and the test should commence as soon as possible but must be within 5 days (120 hours) of collection.

b) Laboratory Qualification

Recognition of the test result by the Ministry of the Environment is dependent upon the test being conducted in a laboratory licenced under the Animals for Research Act, administered by the Ontario Ministry of Agriculture and Food, Veterinary Services Branch.

c) Test Requirements

(i) Rainbow trout (*Salmo gairdneri*) are to be used as the test species.

(ii) Only healthy stocks of acclimated fish as described in section (d) are to be used.

(iii) Individual fish are to weigh between 0.5 and 5.0 grams and the length of the largest fish should not be more than two times the smallest in the same test.

(iv) The water used to dilute the effluent sample must meet all water quality objectives as outlined in Table 1 of the Ministry's publication "Water Management Goals, Policies, Objectives and Implementation Procedures". In addition, dilution water must contain less than a 30 µg/L concentration of total residual chlorine and be capable of maintaining healthy fish stocks for at least ten consecutive days in accordance with item (d).

(v) A concentration range of the bioassay sample must be tested in order to calculate a 96-hour LC50 value. An appropriate logarithmic dilution series should be used which will permit the calculation of 95% confidence limits for the LC50 value using statistically valid methods. A control solution consisting of a 100% concentration of dilution water must also be included in the test.

(vi) For every one gram of fish, there must be at least 0.5 litres of test solution for every 24 hours the fish are exposed.

(vii) The test fish should not be fed within the 24-hour period prior to nor during the test.

(viii) An equal number of fish should be introduced into each test solution at the start of the test. There should be ten fish exposed to each test solution with a minimum of six fish per concentration required for a test to be considered valid.

(ix) Test containers must be non-toxic, inert and non-abrasive.

(xi) A test temperature of 15 +/- 1°C should be maintained and must not deviate from the range of 13-17°C.

(xii) The dissolved oxygen concentration of the test solutions must be measured prior to starting the bioassay. If the dissolved oxygen levels of a test solution is less than 7.0 mg/L, it should be pre-aerated for a minimum period, not to exceed two hours; in order to raise the dissolved oxygen levels to 7.0 mg/L. The rate of aeration must be within the range of 5.0-7.5 cc/min/L.

(xiii) The fish should be introduced to each test solution immediately after the dissolved oxygen concentration is measured to be 7.0 mg/L or more, or immediately after the maximum pre-aeration time limit has expired. The time of introduction of fish should be recorded for each test solution.

(xiv) All fish in a test must be taken from the same fish stock. A random selection of fish from that stock must be distributed among the test solutions.

(xv) An aeration rate of 5.0 to 7.5 cc/min/L should be applied to the test and control solutions throughout the test period.

(xvi) The light regime during the test must be identical to conditions during the holding period (see item d,iv).

(xvii) Observations for fish mortality should be undertaken and recorded as often as possible, with mandatory observations at test times of 1/2, 1, 2, 4, 24 hours and at increments of 24 hours thereafter, including a 96-hour final observation. Dead fish should be removed immediately upon observation. A fish is considered dead when, upon mild mechanical prodding, there is no visible respiratory or other movement. A record should be kept of fish that lose equilibrium but are not dead within the 96-hour time period. If the fish in a test solution appear under stress and recover during the test, an observation to that effect should be recorded.

(xviii) The test is rendered invalid if more than 10% of the fish in the control test solution die within 96 hours.

(xix) All control fish must be sacrificed, weighed and measured (total length) at the termination of the test. All remaining test fish must be sacrificed and disposed of according to d(i).

d) Test Fish

(i) Procurement of Fish Stocks

Stocks of healthy rainbow trout for use in these tests must be certified as free of disease or disease agents as described in Schedule B of the Fish Health Protection Regulations under the Fisheries Act of Canada. This may be accomplished by the purchase of cultured fish from one of the numerous licenced and certified disease free hatcheries operating in Ontario. Otherwise individual stocks of fish must be bacteriologically and virologically diagnosed to determine them as disease free. The following pathogens must not be found in the test fish.

a) Viral Hemorrhagic Septicemia (Egtved) (Egtved virus, VHSV).
b) Infectious Hematopoietic Necrosis (IHNV)
c) Infectious Pancreatic Necrosis (IPNV)
d) Whirling Disease (Myxosoma cerebralis)
e) Ceratomyxosis (Ceratomyxa shasta)
f) Furunculosis (Aeromonas salmonicida)
g) Bacterial Kidney Disease (KD bacterium)
h) Enteric Redmouth Disease (RM bacterium)

At no time must bioassay fish stocks be exposed to prophylaxis or disease treatment (e.g. formalin, malachite green, antibiotics). All diseased fish must be destroyed and sanitarily disposed of, so as not to spread infectious pathogens into receiving waters. Incineration, autoclaving and other sterilization techniques may be used.

(ii) Holding and Acclimation of Fish

Fish should be acclimated to the laboratory conditions for a period of at least 10 days prior to testing and during this time they must be actively feeding and appear as "healthy" (e.g. no abnormal behaviour, no lesions or excessive mucus). Cumulative mortalities during the acclimation and holding period must be less than 1% for the 4 days prior to testing.

Monitoring and assessment of parameters such as pH, chlorine, ammonia, temperature, dissolved oxygen in the holding waters should be performed as frequently as necessary to ensure good quality. Acceptable concentration levels for the maintenance of healthy fish stocks are discussed in the explanatory note in the Biological Test Manual, Ontario Ministry of the Environment (in prep.).

(iii) Holding Facilities

The acclimation tanks and accessories must be made of non-toxic, non-abrasive materials and should be located away from any physical disturbances, preferably in a location separate from the test area. Tanks should be cleaned regularly by scrubbing of the surfaces and siphoning of the settled material.

During the acclimation and holding of fish a constant flow of water through the holding tanks is necessary. Rates of water exchange in holding tanks must be a minimum of 1.4 L/g of fish per day.

(iv) Photoperiod

Natural lighting would be preferable, however, if artificial lighting is used the photoperiod should be representative of the geographical location. To account for seasonal variation, the photoperiod should range from a minimum of 9 hours to a maximum of 15 hours of continuous light and the corresponding continuous hours of darkness. Light intensity at the water surface should be 20 to 30 lux and be of natural light spectrum quality.

(v) Temperature

Since the temperature of the holding water used during the transfer of fish from the hatchery to the laboratory may be outside of the testing limits (13-17 ^{o}C), it may be necessary to gradually change the holding water temperature. The temperature may be changed at a rate not to exceed 1^{o}C per hour and for a total of $5^{o}C$ per day until the testing temperature is achieved. Fish should then be held for 10 days at this temperature but must be held for a minimum of 5 days to complete the acclimation procedure. If during this period, the temperature fluctuates outside the range of $13^{o}C$ to $17^{o}C$, the acclimation is invalidated and must be repeated.

TOXICITY TEST REPORT REQUIREMENTS

The following information must be in the toxicity test report.

(i) Sample

- -name
- -method of collection
- -location
- -collector
- -date of collection
- -collection time
- -chemistry profile (pH, conductivity
- -other chemical parameters may be required depending on the industry sampled.

(ii) Test Conditions

- -laboratory identification
- -date of test
- -test (static, semi static, constant flow)
- -temperature range
- -test volume
- -fish source
- -fish acclimation schedule
- -light regime
- -individual fish lengths (control)
- -individual fish weights (control)
- -holding and dilution water quality (pH, alkalinity, hardness, Ca, Mg, K, Na, Cl, conductivity, metals (Cu, Zn, Pb, Ni, Cd), NH_3, and total residual chlorine.

(iii)

- -LC50 and 95% confidence interval and method of calculation
- -raw mortality dates (times to death)
- -mortality curve
- -loading rate of the bioassay solutions (liters of solution/cumulative fish weight/days of a test)
- -pH, dissolved oxygen and conductivity of test solutions at start of test and in a test solution at the time of 50% mortality.

(iv)

- -indications of stress.